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Ablation of experimental colon cancer by intratumoral ^{224}Radium-loaded wires is mediated by alpha particles released from atoms which spread in the tumor and can be augmented by chemotherapy

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Abstract

Purpose: **We developed ^{224}Ra-loaded wires, which release by recoil alpha emitting nuclei into solid tumors and cause tumor cell killing. This research examined if the major damage was inflicted by alpha particles emitted from these atoms or by direct gamma and beta emissions from the inserted wires. We also examined the efficacy of this treatment against colon cancer in combination with chemotherapy.**

Materials and methods: **Mouse colon carcinomas (CT-26 xenografts), treated by intra-tumoral radioactive wires loaded with ^{224}Ra atoms were monitored for effects on tumor growth, intratumoral tissue damage and distribution of alpha emitting atoms. The effects were compared with those of ^{224}Ra-loaded wires coated with poly methyl methacrylate (PMMA), which blocks atom recoil. Similar experiments were performed with radioactive wires combined with systemic 5-FU.**

Results: **^{224}Ra-loaded wires inhibited tumor growth and formed necrotic areas inside the tumor. PMMA coated wires did not inhibit tumor growth, and caused minor intratumoral damage. Autoradiography images of tumors treated with ^{224}Ra-loaded wires revealed a spread of alpha emitters over several mm, whereas PMMA-coated wires showed no such spread. Injection of 5-FU with ^{224}Ra-loaded wires augmented tumor growth retardation and cure.**

Conclusions: **^{224}Ra-loaded wires ablate solid tumors by the release of alpha-particle emitting atoms inside the tissue, an effect that can be enhanced by combining this method with chemotherapy.**

Keywords: Colon cancer, radiotherapy, chemotherapy, 5-FU, ^{224}Ra, brachytherapy

Introduction

Ionizing radiation causes many types of DNA damage, including base damage and single- and double-strand breaks. Photons, namely X-rays and gamma-rays, are the most widely used type of ionizing radiation in radiobiology experiments and in radiation cancer therapy. Their use in external beam radiation therapy (EBRT) is the standard care for almost 70% of cancer patients providing a non-invasive method for delivering the radiation to the tumor. Radiation can be also delivered in the form of Brachytherapy, which uses intratumoral implants emitting mostly gammas and X-rays (Williamson 2006).

The use of charged particles, including protons, alpha particles, or heavy high-energy particles such as carbon ions and iron, is currently very limited. The physical and radiobiological basis of the action of energetic, high-LET charged particles suggest that their use can represent a technical improvement for conformal therapy of radiotherapy-resistant cancers such as renal-cell carcinoma (Nomiya et al. 2008) melanoma (Li et al. 2007) and glioblastoma (Combs 2009) (for review see also Durante and Loeffler 2010, Loeffler and Durante 2013).

Heavy ions in the Bragg peak region induce cell killing independently of free radicals or reactive oxygen species, via direct induction of complex, clustered damage to DNA (Cucinotta and Durante 2006) whose enzymatic repair is particularly difficult. Besides, the efficacy of cell killing by heavy ions is largely independent of DNA repair mechanisms involving TP53 or BCL2 status (Hamada 2009).

Alpha particles (6–9 MeV and LET of 100–200 keV/micron) have a high relative biological effectiveness (RBE)

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and similar effects as heavy ions. Only few hits are required to ensure cell lethality (Søyland and Hassfjell 2000, Suntharalingam et al. 2005, Roeske and Stinchcomb 2006). As with heavy ions, the effect of alpha particles is less sensitive to the cell oxygenation state compared to photons and electrons (Tobias et al. 1982, Antonovic et al. 2013, Tinganelli et al. 2013).

Yet, alpha particles, because of their short range in tissue (40–90 micron), are not in use for external beam or brachytherapy of solid tumors. Alpha radiation is rather used in radionuclide therapy by combining radionuclides with cancer targeting agents, such as antibodies, hormones, or custom-designed synthetic agents to multifocal disease (Couturier et al. 2005, Bruland et al. 2006, Brechbiel 2007, Huang et al. 2012).

Our method, termed as DaRT – Diffusing Alpha-emitters Radiation Therapy – is the first approach for treating solid tumors with alpha particles in brachytherapy.

DaRT entails treating solid tumors with interstitial ^{224}Ra-loaded wires that continually release short-lived alpha emitting atoms into the tumor. Wire preparation is based on a ^{228}Th (1.91 y half-life) generator (Arazi et al. 2007). The wires, impregnated with small activities of ^{224}Ra (3.66 d half-life) embedded securely below their surface, are inserted into the tumor, releasing, by recoil, ^{220}Rn, ^{216}Po and ^{212}Pb atoms. These atoms disperse to a considerable distance from the source in the tumor, leading to the formation of a high dose alpha radiation region (Arazi et al. 2007). Alpha emitting atoms which get out of the tumor enter the blood system and disperse in the entire body delivering a very low dose to most organs and reduced damage to the immediate vicinity of the tumor (Arazi et al. 2010).

The ^{224}Ra decay chain involves, along with the emission of alpha particles, also several beta and gamma emissions. Beta decays occur for ^{212}Pb (with a maximum energy $E_{max} = 570$ keV), ^{212}Bi ($E_{max} = 2254$ keV) and ^{208}Tl ($E_{max} = 1796$ keV). The most important gamma emissions occur for ^{212}Pb (239 keV, branching ratio 43.6%) and ^{208}Tl (511 keV, 22.6%; 583 keV, 84.5%; 861 keV, 12.4%; 2615 keV, 99.2%), with additional lines at lower energies (< 90 keV) with branching ratios of up to 17%. The effective range of beta particles with the above energies in tissue is a few mm. The attenuation lengths of the dominant gamma emissions are 8–23 cm, indicating that the dose contribution from these gammas falls roughly as $1/r^2$ in the vicinity of the source.

In the present study, we tested the biological effect of this alpha particle based therapy on colon cancer derived tumors in mice. We assessed the separate contributions of the alpha radiation from the atoms which spread inside the tumor and of the betas and gammas emitted directly from the ^{224}Ra source, to tumor damage. We also examined the combined effects of the radioactive wires with the chemotherapy 5-fluorouracil (5-FU).

Materials and methods

Animals

Balb/c male mice were obtained from the breeding colony of Tel-Aviv University, Israel. Mice were used at the ages of 8–12 weeks. Animal care and experimentation was carried out in accordance with Tel-Aviv University guidelines (Permit No. M-07-060, M-10-059).

Tumor cell lines

CT26 cells, an N-nitroso-N-methylurethane (NNMU)-induced, undifferentiated colon carcinoma cell line was purchased from the ATCC (CRL-2638). CT26 cells were grown in Roswell Park Memorial Institute medium (RPMI-1640, GIBCO, Rehovot, Israel) supplemented with 10% fetal calf serum, L-glutamine (2 mM), sodium pyruvate (1 mM), Hepes buffer 1M and D-glucose (Biological Industries, Beit Haemek, Israel), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Sigma, Paisley, USA), The cell lines were stored in a humid incubator at a temperature of 37°C, CO_2 5%.

Chemotherapeutic agent

5-Fluorouracil (5-FU) was purchased from Ebewe Pharma (Unterach, Austria), kept at room temperature and dissolved in PBS before use.

Tumor cell inoculation

Subcutaneous tumors were induced by injecting a single dose, i.d. of 5×10^5 CT26 cells in 0.1 ml HBSS buffer (Biological industries, Israel) into the low lateral side of the Balb/c mice back (Plotnikov et al. 2004). Tumors appeared after 10 days (3–4 mm).

Local tumor growth was determined by measuring three mutually orthogonal tumor diameters with a digital caliper. The volume of the tumor was calculated as: $V = D_1 \times D_2 \times D_3 \times (\pi/6)$, where D_1, D_2, D_3 stand for the three mutually orthogonal tumor diameters.

^{224}Ra-loaded wire preparation

^{224}Ra-loaded wires were made of 0.3 mm diameter stainless steel acupuncture needles (Golden Needle, China) or titanium wires (0.508 mm in diameter, Grade 1 titanium, New England Precision Grinding Co. Holliston, MA, USA). Wires were loaded with radium by exposing them to a ^{228}Th generator in an electrically isolated, air-filled circuit as previously described (Cooks et al. 2008). In order to embed the accumulated radium ions into the wire's surface and thus prevent them from being removed once inside the tissue, the wire was heated in N_2 atmosphere to about 500°C to induce diffusion of radium from the surface to a depth of 1–20 nanometers, which still allows considerable recoil of the alpha emitting daughters into the tumor. The ^{224}Ra activity and release rate of ^{220}Rn of ^{224}Ra-loaded wires were then characterized by an alpha particle detector (Cooks et al. 2008, Horev-Drori et al. 2012). The wires were inserted into the tumors a short time after their preparation. The wires were made in the School of Physics and Astronomy, Faculty of Exact Sciences, Tel Aviv University.

Preparation of PMMA-coated wires

To separately test the contribution of betas and gammas from the wires, radioactive wires were coated with PMMA in order to prevent the recoil of the daughter atoms of ^{224}Ra into the

tumor. Coating was performed by dipping the wires in PMMA solution (10% in Methyl isobutyl ketone, Bio-Lab Ltd).

DaRT wire implantation

Immediately after a DaRT wire was prepared and its activity measured (^{224}Ra activity; 7–65 kBq), it was placed at the extremity of a 23G syringe (PiC Indolor, Italy), which had a conductor wire inside it. Thus, it was possible to penetrate to a desired depth of the tumor using the syringe and subsequently manipulate the conductor to leave the DaRT wire inside the tumor.

Anesthesia

Anesthetic compound (100 mg/kg ketamine + 10 mg/kg xylazine hydrochloride solution) (Vetoquinol, Eurovet), intra-peritoneal inoculation of 0.25 ml (solution in PBS) was given 10 min before starting the treatment. All surgical and invasive procedures were carried out under anesthesia.

Histology

CT26 tumors were taken for histological examination at different times after DaRT treatment. Immediately after their removal, specimens were put into 4% formalin solution for at least 24 h. The preserved specimens were embedded in paraffin; histological sections (5 or 10 μm) were cut using a Leica RM2055 microtome (Leica, Nussloch, Germany, Microtome blades, Feather S35 Type), and placed on glass slides. The samples were stained with hematoxylin-eosin (H&E) (Surgipath, Richmond, IL, USA) and analyzed for tissue damage.

The following reagents and equipment were used for tissue processing: Formaldehyde, 37%/wt. (Aldrich, 533998 – 500 ml), Ethanol 70% (Gadot, #L70067608), Ethanol absolute (Bio Lab, #05250504), Toluene (Bio Lab, #20150501), Paraffin no. 1 = paraplast plus (McCormick 502004), Paraffin no. 2 = tissue wax, Hematoxylin (Surgipath – Harris Formula, #01562), Hydrochloric acid 32% (Merck), Eosin (Surgipath, #01600), Entellan (Merck, HX1.07961.0500), Xylene (Gadot, #L70066734).

High resolution autoradiography (HRA) of DaRT-treated tumors

Excised tumors were placed in 4% formaldehyde for duration of 24–36 h. Shortly after tumor excision the DaRT wire was removed (by retracting it along its axis), to prevent further build-up of ^{212}Pb inside the tumor. The preserved specimens were processed and embedded in paraffin following standard procedures. Histological sections (8 μm) were cut using a Shandon Finesse microtome (Thermo, Cheshire, UK) perpendicularly to the wire axis and placed on glass slides. The slides were then laid on a Fuji phosphor-imaging plate (BAS-TR2040S, Fuji Photo Film, Japan) over a 12 μm Mylar foil to prevent direct contact with the plate, recording a signal proportional to the local ^{212}Pb activity of the histological sections (Arazi et al. 2007); this signal is dominated by the contribution of alpha particles emitted by ^{212}Bi and ^{212}Po (in secular equilibrium with ^{212}Pb), with negligible contribution from betas and gammas. Slides were left for measurement on the phosphor-imaging plate for about 10 h in a light-tight box. The plate was scanned by a Fuji FLA-9000 system with a pixel size of 100 μm. The images produced were then processed using the TINA software (Raytek, Sheffield, UK) to find the total photo-stimulated luminescence (PSL) values (minus the background) in regions of interest of equal areas surrounding the tumor slices.

An optional further layer in the analysis consisted of estimating the alpha particle dose based on the measured PSL patterns. The procedure, described in detail in Arazi et al. (2007), begins by converting the PSL value of each pixel to the local ^{212}Pb activity inside the tumor section (just above the pixel), using a set of ^{212}Pb calibration samples measured on the Fuji-plate simultaneously with the tumor sections. This provides a direct estimate of the local ^{212}Pb activity at the time of tumor excision. The local alpha particle dose of ^{212}Bi and ^{212}Po (which are essentially in secular equilibrium with ^{212}Pb at all times) is proportional to the time integral of the local ^{212}Pb activity. This integral is calculated by assuming that the spatial pattern of ^{212}Pb activity is approximately constant throughout the treatment, with a time-dependent factor which describes its initial buildup and subsequent decay with ^{224}Ra half-life [Arazi et al. 2007]. Following this mathematical procedure, one can obtain rough estimates for the ^{212}Bi and ^{212}Po alpha particle dose distribution from the time of treatment (wire insertion) until the time of tumor excision, as well as the asymptotic dose that would have been delivered over an infinite time (in practice – over several ^{224}Ra half-lives). One can thus roughly quantify the treatment by calculating the effective diameter of the area receiving a dose above some set value (e.g., 10 Gy). Note that these calculations relate to only one of the three alpha particles emitted for each ^{220}Rn atom recoiling from the wire into the tumor. The dose contributed by the alpha decays of ^{220}Rn itself and of its immediate daughter ^{216}Po, is concentrated close to the wire, in a region where the alpha particle dose of ^{212}Bi and ^{212}Po is already high enough in itself to ensure cell lethality.

Statistical analysis

The statistical significance ($p \leq 0.05$) of the differences between volumes of tumors in the various groups was assessed by applying ANOVA: Two-factor without replication or Student's *t*-test with repeated measures, by SPSS or Excel software.

Results

Ablation of colon tumors in Balb/c by intra-tumoral ^{224}Ra-loaded wires

In order to examine the effect of ^{224}Ra-loaded wires on tumor growth, one or two ^{224}Ra wires were inserted horizontally along the center of the tumor. Applying two wires per tumor (total 23.7–24.8 kBq) resulted in significant retardation of tumor growth compared with tumors in which two inert wires were inserted (Figure 1, $p = 0.014$). Twenty-nine days post treatment, the average volume of the ^{224}Ra-loaded wires treated tumors ($n = 6$) was 3 times smaller than that of the inert wires treated group ($n = 7$, Figure 1). Two wires achieved a better local tumor control compared to the group treated with one wire (11.7–12.3 kBq) that release lower radiation

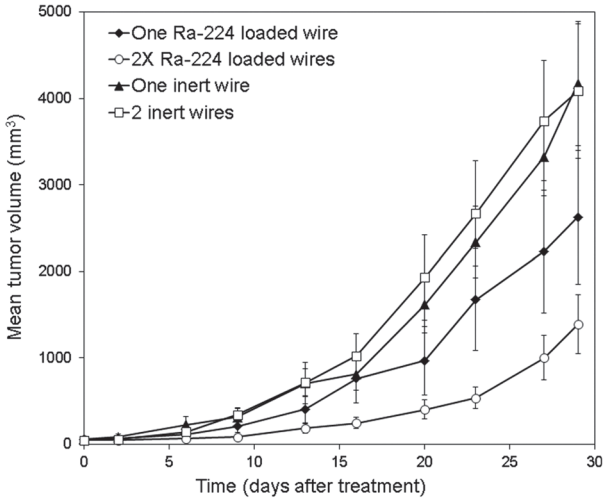

Figure 1. Effect of treatment with ^{224}Ra-loaded wires on the development of murine colon tumors. Treatment was applied to Balb/c mice bearing tumors (5–6 mm average diameter) and monitored for tumor growth. One ^{224}Ra wire: Tumor-bearing mice treated each with one ^{224}Ra wire, carrying activity in the range of 11.7–12.3 kBq ($n = 6$). Two ^{224}Ra wires: Tumor-bearing mice treated with 2 ^{224}Ra wires, carrying total activity in the range of 23.7–24.8 kBq ($n = 6$). Inert: Tumor-bearing mice treated with one inert wire ($n = 5$). 2 × Inert: Tumor-bearing mice treated with 2 inert wires ($n = 5$). *P*-values (ANOVA without replication) of Two ^{224}Ra-loaded wires vs. one ^{224}Ra-loaded wire or one/two inert wire(s) $p < 0.05$, or one ^{224}Ra-loaded wire vs. one/two inert wire(s) $p < 0.05$.

activity, but the differences were not statistically significant (Figure 1, $p = 0.782$).

As noted above, apart from alpha particles, the ^{224}Ra decay chain consists of both beta and gamma emissions, which might further contribute to tumor cell eradication. To evaluate such additional contribution emitted from DaRT wires we compared the effect of ^{224}Ra-loaded titanium wires to the effect of ^{224}Ra-loaded wires coated with Poly methyl methacrylate (PMMA). PMMA blocks the recoil of alpha emitting radioactive atoms from the loaded wires, allowing only the beta-particles and gamma-rays to leave the wire, thus separating the damage caused by the different sources of radiation. Tumors (6–7 mm in diameter) were treated either with a single DaRT wire (average ^{224}Ra activity 44.3 kBq), an inert wire or a DaRT wire coated with PMMA (average ^{224}Ra activity 42.7 kBq) ('sealed' wire). Tumors treated by DaRT showed the lowest growth rate compared to tumors treated with either inert or PMMA-coated wires. Tumors treated with inert or PMMA-coated wires presented almost the same growth progression curves (Figure 2A). Histological examination of treated tumors, taken out 5 days post wire insertion, revealed that active wires caused tumor necrosis, which was absent in tumors treated with PMMA-coated wires (Figure 2B). In order to verify that coated radioactive wires do not release alpha emitting atoms into the tumor, we measured the distribution of alpha emitters (^{212}Bi and ^{212}Po) inside the tumors 4 days post treatment using the Fuji phosphor-imaging plate. The results presented graphically in Figure 3A, were calculated from alpha particle distribution around radioactive wires (Figure 3B), and around PMMA-coated wires (Figure 3C). In the case of PMMA-coated wires, the active region was limited to the immediate vicinity of the wire (~ 1 mm), and

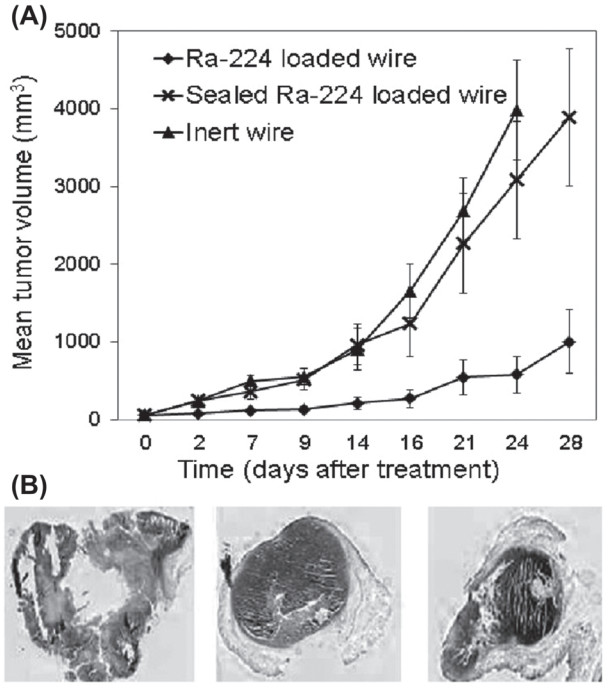

Figure 2. Effect of blocking the recoil of atoms from ^{224}Ra-loaded wire on tumor destruction. Treatment was applied to Balb/c mice bearing tumors (6–7 mm average diameter) and monitored for tumor growth. ^{224}Ra wire: Tumor-bearing mice treated with one ^{224}Ra wire, carrying activities in the range of 41–47.2 kBq ($n = 8$). Sealed: Tumor-bearing mice treated with one PMMA-coated ^{224}Ra wire, carrying activities in the range of 38.1–46.5 kBq ($n = 7$). Inert: Tumor-bearing mice treated with an inert wire ($n = 7$). *P*-values of (ANOVA – two-factor without replication and Tukey test), ^{224}Ra-loaded wire vs. sealed or inert < 0.05 (A). H&E staining of CT-26 tumors treated by a ^{224}Ra-loaded wire (left), sealed (middle) or inert wire (right), 5 days post treatment (B).

had a total PSL signal smaller by a factor of ~ 80 compared to that of the uncoated wires.

We further calculated the asymptotic alpha particle dose distribution, resulting from the decay of ^{212}Bi and ^{212}Po inside

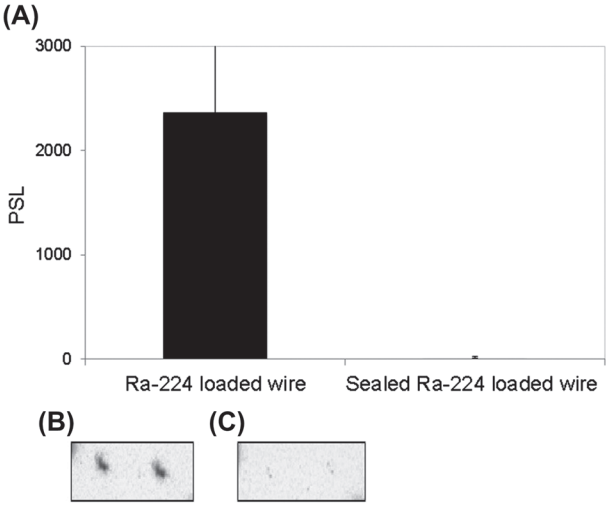

Figure 3. Spread of radioactive atoms inside the tumors. Tumors (6–7 mm in average diameter) were treated by one ^{224}Ra-loaded wire, carrying activities in the ranges of 41.4–47.5 kBq ($n = 3$) or PMMA-coated ^{224} Ra-loaded wires (sealed) carrying activities in the ranges of 37–48.4 kBq ($n = 4$). Four days post wire insertion the distribution of alpha particle emitting atoms was monitored by PSL (Photostimulated Luminesence). *P*-value (*t*-test) = 0.007 (A). Tumor slices of ^{224}Ra-loaded wire (B), or treated with one PMMA-coated ^{224}Ra-loaded wire (C).

the tumor (Figure 4) as described above, based on an autoradiography image of a 10 micron-thick section taken from the treated tumor (a different tumor than the one shown in Figure 3). The tumor was treated 11 days after tumor cell inoculation, with a single DaRT wire, with a ^{224}Ra activity of 31 kBq, inserted parallel to the tumor base. The tumor's long diameter at the time of treatment was ~ 7 mm. The tumor was removed 3.9 days after source insertion. As can be seen in the Figure, an asymptotic alpha particle dose exceeding 10 Gy is expected over a region spanning 3–4 mm. The central dip observed in the calculated dose map resulted from tissue deterioration in the immediate vicinity of the DaRT wire.

The calculated dose is consistent with the wire activity, assuming an effective diffusion coefficient of the order of 10^{-8} cm^2/s for ^{212}Pb (which likely binds to large molecules and does not diffuse as a free ion). In the context of a mathematical diffusion model, as given in the appendix of Arazi et al. (2007), the effective diffusion length of ^{212}Pb in this case is ~ 0.3 mm. Note that separate Monte Carlo calculations of the dose delivered by betas and gammas (emitted both by the source and by the diffusing atoms), show that for this wire activity, the asymptotic beta dose is larger than 10 Gy up to ~ 2 mm from the wire, i.e., overlapping the region receiving > 10 Gy from the alpha particles emitted by ^{212}Bi and ^{212}Po. The gamma contribution to the dose in this case falls below 1 Gy at distances larger than ~ 1 mm from the wire and is thus negligible.

Ablation of colon tumors in Balb/c mice by intra-tumoral ^{224}Ra-loaded wire combined with 5-FU

We examined the therapeutic efficacy of 5-FU in combination with the radioactive wires on colon tumor retardation. Randomized mice with 6–7 mm (longest diameter) tumors were divided into four groups and subjected to one of the following treatments: Two ^{224}Ra-loaded wires per tumor (total radioactivity 27.9–35.5 kBq), two inert wires, two ^{224}Ra-loaded wires per tumor (32.1–33.8 kBq) combined with 5-FU (75 mg/kg) injected 24 h prior to wire insertion, or two non-radioactive wires per tumor combined with 5-FU (75 mg/kg).

The results presented in Figure 5 demonstrate that treatment with two ^{224}Ra-loaded wires inserted concomitantly with a single dose of 5-FU, significantly retarded tumor growth compared to the effect of radioactive wires alone ($p = 0.0004$) or inert wires + 5-FU ($p = 0.015$) (ANOVA: Two-factor without replication). Furthermore, in 4 out of 5 mice the tumor completely disappeared after DaRT + 5-FU while only 1 out of 5 disappeared after DaRT alone. The mice whose primary tumor was eradicated by DaRT + 5-FU were alive 40 days after treatment without tumor recurrence.

Similar results were obtained in mice treated with a lower radioactive dose (one wire 7.2–19 kBq) ($n = 13$) without and with 5-FU (75 mg/kg). When treated with the combination of ^{224}Ra-loaded wire and 5-FU, better local tumor control was achieved, compared with the group treated with ^{224}Ra-loaded wire alone ($p = 0.01$) or inert wire + 5-FU ($p = 0.033$) (ANOVA: Two-factor without replication).

Discussion

Colon cancer accounts for 9% of all new cancer cases and is the third leading cause of cancer death in men and women in the USA (Siegel et al. 2013). Surgery remains the cornerstone of successful treatment of gastrointestinal carcinomas. However, strategies adding chemotherapy, radiation therapy or both have evolved and were proven to reduce relapse rates. Radiotherapy (RT) alone used in either a pre-operative or a post-operative setting improves the overall survival compared to surgery alone (Oehler and Ciernik 2006).

DaRT (Diffusing Alpha-emitters Radiation Therapy) is a new method for treating solid tumors with interstitial

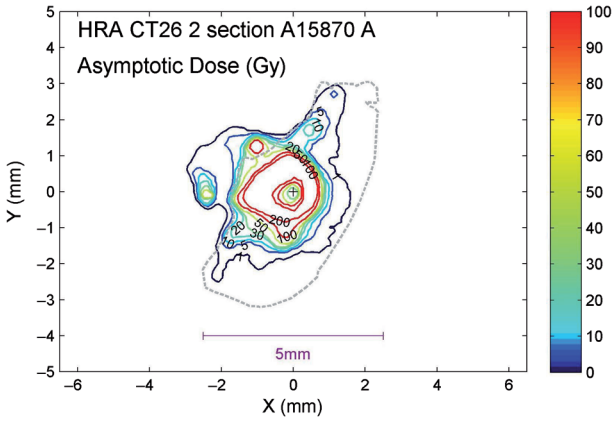

Figure 4. Calculated asymptotic dose contours (Gy) based on autoradiography measurements with a Fuji phosphor-imaging plate, in 10 µm histological sections taken from tumors. The scale refers to the alpha particle dose delivered by ^{212}Bi and ^{212}Po from source insertion until tumor removal (Gy). The nominal source ^{224}Ra activity at the time of insertion was 0.84 µCi (31 kBq). Dose curves shown: 1, 5, 10, 20, 30, 50, 100, 200 Gy. The border of the tumor is marked by a dashed line. This Figure is reproduced in color in the online version of *International Journal of Radiation Biology*.

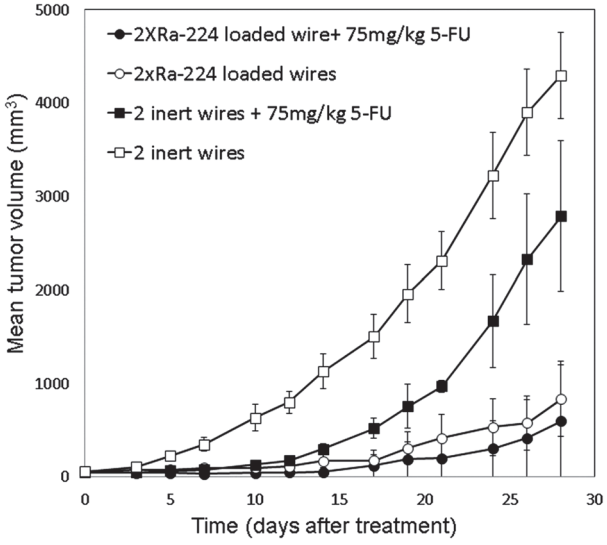

Figure 5. Effect of treatment with ^{224}Ra-loaded wires combined with 5-FU on the growth of murine colon cell derived tumors. Balb/c mice bearing 6–7 mm in diameter subcutaneous tumors were treated with either two ^{224}Ra-loaded titanium wires (total activity 27.9–35.5 kBq; $n = 5$), two ^{224}Ra wires (total activity 32.1–33.8 kBq) combined with 5-FU ($n = 5$), two inert wires combined with 5-FU ($n = 6$), or two inert wires ($n = 6$). 5-FU (75 mg/kg) was injected 24 hours prior to wire insertion. *P*-value (ANOVA two-way without replication) of two ^{224}Ra-loaded wires + 5-FU vs. all controls < 0.05.

radioactive wires that continually release short-lived alpha particle emitting atoms into the tumor (Arazi et al. 2007). In this research study we explored the effect of DaRT as a stand-alone treatment or in combination with chemotherapy on colon cancer.

Insertion of one ^{224}Ra-loaded wire (11.7–12.3 kBq per wire) into CT26 tumors (5–6 mm in diameter) had a pronounced retardation effect on the progress of tumor growth compared to inert wires (62% of control). The insertion of two ^{224}Ra wires carrying a total activity of 23.7–24.8 kBq achieved a higher level of tumor destruction (33% of control), which indicated that tumor ablation is dependent on the amount of radioactive atoms which spread in the tumor, and on the geometrical coverage by the DaRT wires.

The ^{224}Ra-loaded wires used in this study emit beta and gamma radiation over a wide range of energy. Thus it was important to ensure that the damage caused to the tumor is derived primarily from the alpha particles emitted by the diffusing atoms and not from the direct beta and gamma emissions from the wire. To test this we compared the damage inflicted by a DaRT wire to that of a radioactive wire coated with PMMA to prevent the recoil and spread of radioactive atoms inside the tumor. Tumors treated by DaRT wires were significantly smaller than tumors treated by PMMA-coated or inert wires. There was no significant difference between the volumes of tumors treated by an inert wire or a PMMA-coated wire (Figure 2A). Histological examination of tumor slices 5 days after treatment revealed considerable necrotic areas caused by DaRT wires, while very limited damage was seen in tumors treated by PMMA-coated radioactive or inert wires (Figure 2B).

Using high-resolution autoradiography it was shown that radioactive atoms (namely ^{212}Po and its daughters) spread over several mm in tumors treated by DaRT wires (Figure 3B) but not in tumors treated by PMMA-coated wires (Figure 3C).

Autoradiography-based dose estimations for treated CT-26 tumors show that the region receiving an asymptotic alpha-particle dose exceeding 10 Gy (by ^{212}Bi and ^{212}Po) is 3–4 mm in diameter. Interestingly, the dose contribution of beta emissions from the wire and diffusing atoms is similar over the same region (while the contribution from gammas is negligible). The observation that PMMA-coated wires did not have an effect on tumor growth thus suggests that in spite of the similar doses, the biological effect of the diffusing alpha emitters is much larger. One possible explanation is that the radiobiological effectiveness (RBE) of alpha particles is considerably higher than that of betas (for which RBE = 1) in these tumors. Note that in a previous microdosimetric in vitro study (Lazarov et al. 2012) we have found that the survival probability for CT-26 cells whose nucleus is hit by a single alpha particle is 0.65 ± 0.12, one of the lowest values obtained for the several cell lines investigated.

In previous studies we have also demonstrated that DaRT inhibited tumor development, extended survival, and reduced lung metastases in mice bearing Squamous cell carcinoma (Arazi et al. 2007, Cooks et al. 2008), lung carcinoma (Cooks et al. 2009a), and pancreatic carcinoma (Horev-Drori et al. 2012) derived tumors. DaRT was also effective against human tumors transplanted into athymic mice (Cooks et al. 2012). It was observed that tumors of different histotypes responded differently to the treatment (Lazarov et al. 2012).

The tumor response to radiation is determined not only by the tumor cell phenotype but also by the microvascular sensitivity. Tumors grown in apoptosis-resistant mice displayed markedly reduced baseline microvascular endothelial apoptosis, exhibited reduced endothelial apoptosis upon irradiation and were more resistant (Garcia-Barros et al. 2003). The results for experimental tumors strongly indicate that radiation of human tumors with high doses may induce considerable vascular damages and thereby leading to indirect tumor cell death (Park et al. 2012). In a previous study we observed that tumor destruction by alpha radiation is achieved by direct damage to tumor cells and by damage to the vasculature (Cooks et al. 2008).

Since recurrence of treated tumors depends on angiogenesis, it is thus important to take into consideration that heavy charged particles can affect both the tumor cells and the tumor microenvironment. It has been shown that densely ionizing radiation elicits signalling pathways quite distinct from those involved in the cell and tissue response to photons (Durante 2014). To this effect, it has been demonstrated that high-energy proton irradiation can inhibit expression of major pro-angiogenic factors and multiple angiogenesis-associated processes, including invasion and endothelial cell proliferation, and thus achieve better inhibition of cancer progression (Girdhani et al. 2012).

As DaRT is based on alpha-particles, it should be particularly powerful against cancer stem cells (CSCs) which were reported to be more resistant to sparsely ionizing radiation (X-rays or gamma-rays) than their stem-cell counterparts (Baumann et al. 2008). It was already suggested that radiotherapy with high-energy charged particles should be considered to specifically target CSCs (Diehn et al. 2009, Durante and Loeffler 2010, Pignalosa and Durante 2012).

Several approaches have proven effective in sensitizing cells to the killing effects of radiation, including hyperthermia, small molecule drugs or genetic approaches to inhibit DNA repair, and oxygen increase (Harrison et al. 2002, Vallerga et al. 2004, Iliakis et al. 2008).

Some chemotherapeutic drugs destroy tumor cells by their own cytotoxic action and additionally enhance the effects of low-LET radiotherapy. Chemotherapeutic drugs that have the potential to produce substantial sensitization of tumor cells to gamma radiation treatment are defined as radiosensitizers (Wilson et al. 2006, Shewach and Lawrence 2007). A number of clinical studies have shown that both survival rate and palliative benefit can be improved when radiotherapy is combined with chemotherapy in the treatment of various tumors (Doyle et al. 2001, Crane et al. 2002, Curran 2002, Varveris et al. 2003).

In the present study we examined the potential of radioactive ^{224}Ra-loaded wires in combination with 5-fluorouracil (5-FU) to achieve local control of malignant colon tumors in mice. 5-FU rapidly enters the cell using the same facilitated transport mechanism as uracil. 5-FU is converted intracellularly to several active metabolites which disrupt RNA synthesis and the action of thymidylate synthetase – a key

enzyme converting dUMP to dTMP – which is essential for DNA synthesis and repair. Unfortunately, response rates for 5-FU-based chemotherapy as a first-line treatment for advanced colorectal cancer are only 10–15% (Longley et al. 2003, Makizumi et al. 2008).

Importantly, 5-FU was also reported as radiosensitizer (Shewach and Lawrence 2007) when given in combination with gamma radiation and it was of interest to find out if the combination of alpha radiation and 5-FU will be better than each treatment alone.

We tested the effect of a combination treatment of ^{224}Ra-loaded wires with one time administration of 5-FU on mice bearing tumors. Treatment with a ^{224}Ra-loaded wire with 5-FU was significantly better than a treatment with an inert wire, 5-FU alone or a ^{224}Ra-loaded wire alone. Treatment with two ^{224}Ra-loaded wires and 5-FU had a pronounced retardation effect that further led to complete cure of 4 out of 5 mice. DaRT treatment could also be effectively combined with chemotherapy to treat squamous cell (Cooks et al. 2009b) and pancreatic carcinoma (Horev-Drori et al. 2012). Yet, exposure of tumor cells in vitro to chemotherapeutic drugs did not increase their sensitivity to alpha particles (unpublished results), so we assume that the increase in survival of the animals treated by the combination is due to eradication of lung metastases by the chemotherapy.

The combination of alpha radiation and chemotherapy may also be important for abrogating angiogenesis and vasculogenesis driven by an influx of CD11b$^+$ myeloid cells (macrophages) and endothelial progenitor cells (EPC) into the tumors that contribute to the radioresistance of tumors (for review see Brown 2014).

Lastly, destruction of the primary tumor may also release tumor antigens that will stimulate anti-tumor immunity, which can eliminate residual disease, as was shown to occur after electrochemical tumor ablation (Plotnikov et al. 2004). Thus, combining DaRT with immunostimulation may lead to a novel approach for treating solid metastatic malignancies.

In conclusion, the use of the DaRT method on mouse colon cancer was shown to result in a pronounced local control of the tumor; combining DaRT with chemotherapy yielded the elimination of primary tumors and led to a complete cure of CT26 derived tumors. These results add to those of our previous investigations, which as a whole suggest that DaRT has the potential to become an efficient and safe method for prolonged treatment of the entire volume of solid tumors with therapeutic doses of alpha particles.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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